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Mechanism of Action of Nalidixic Acid on *Escherichia coli*

II. Inhibition of Deoxyribonucleic Acid Synthesis

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ABSTRACT

GOSS, WILLIAM A. (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), WILLIAM H. DEITZ, AND THOMAS M. COOK. Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. **89**:1068-1074. 1965.—Nalidixic acid was shown to inhibit specifically the synthesis of deoxyribonucleic acid (DNA) in *Escherichia coli*. Slight effects on protein and ribonucleic acid (RNA) synthesis were observed only at higher levels of drug or after prolonged incubation. The inhibition of DNA synthesis in *E. coli* 15TAU, as measured by incorporation of C^{14}-labeled thymine, was observed after exposure to nalidixic acid for 10 min. Inhibition of the incorporation of C^{14}-labeled uracil into RNA and C^{14}-labeled L-arginine into protein (21 and 28% inhibition, respectively) was observed only after 60 min of exposure. When cultures of *E. coli* 15TAU were exposed to 3.0 μg/ml of nalidixic acid (slightly greater than the minimal growth inhibitory concentration), the incorporation of C^{14}-labeled thymidine was inhibited 30 to 40% after 90 min. Nalidixic acid at 10 μg/ml, a lethal concentration, inhibited thymidine incorporation 72% during this period. Nalidixic acid at 1.0 μg/ml had no apparent effect on the incorporation of C^{14}-labeled adenine or C^{14}-labeled uracil into RNA of cultures of *E. coli* 198, a wild-type strain. However, incorporation of both bases into DNA was strongly inhibited after 60 min of exposure (66 and 69%, respectively). Nalidixic acid inhibited DNA replication during a single round of synthesis. In contrast with "thymineless death," nalidixic acid was not lethal to *E. coli* 15TAU during restricted RNA and protein synthesis (i.e., in a medium containing thymine but lacking arginine and uracil). We have shown also that this chemotherapeutic agent has little effect on the synthesis of protein or RNA required to initiate DNA replication. After 75 min of inhibition, the capacity of *E. coli* 15TAU to synthesize DNA in a medium containing thymine, arginine, and uracil may be restored by a simple filtration and washing process, indicating that the drug is not firmly bound. These studies leave little doubt that a primary action of nalidixic acid is the inhibition of the synthesis of DNA in *E. coli*.

Previous investigations (Goss, Deitz, and Cook, 1964) have demonstrated that nalidixic acid is lethal for proliferating cultures of *Escherichia coli*. Associated with this lethal effect is the formation of elongated, serpentine forms. Chemical analysis of major cellular constituents revealed that only deoxyribonucleic acid (DNA) levels are markedly lowered in drug-treated cells. These facts are consistent with the view that nalidixic acid interferes with DNA synthesis in *E. coli*.

This communication reports the results of further studies of the mechanism of action of nalidixic acid on *E. coli*. Radioisotopic techniques were used to examine the effect of nalidixic acid on the incorporation of precursors of DNA, ribonucleic acid (RNA), and protein.

MATERIALS AND METHODS

Cultures, media, and the methods of determining bacterial viability were described previously (Goss et al., 1964).

For studies with *E. coli* 15TAU, the terminology of Maaløe and Hanawalt (1961) will be employed to describe nutritional conditions. Thus, (+T, −AU) will refer to a medium containing thymine, but no arginine or uracil; (−T, +AU) refers to a medium containing arginine and uracil but no thymine.

Medium changes. To change growth media or remove supplements, cells were collected by membrane filtration (Millipore, 0.45-μ), washed, and suspended in warm basal medium. Supplements were then added to give the desired medium.

Radioisotopes. The following radioactive compounds were purchased from the New England

Nuclear Corp., Boston, Mass.: uniformly labeled L-arginine-C^{14} (246 mc/mmole), uracil-2-C^{14} (27.8 mc/mmole), thymine-2-C^{14} (45.4 mc/mmole), and thymidine-2-C^{14} (30 mc/mmole). Adenine-8-C^{14} (20 mc/mmole) was obtained from Calbiochem.

Incorporation of radioactive precursors. At intervals after the addition of radioactive precursors, samples were removed to equal volumes of ice-cold 10% trichloroacetic acid. After 30 min at 0 C, the acid-insoluble precipitates were collected by membrane filtration (0.45-μ porosity; Millipore Filter Corp., Bedford, Mass.), and were washed with ice-cold 1% trichloroacetic acid. The membrane and precipitate were dissolved in 1.0 N NaOH, and the radioactivity was measured with a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Corp., Chicago, Ill.). Counting efficiency in this system was 40 to 45%.

The modified Schmidt-Tannhauser technique of Roodyn and Mandel (1960) was employed to study the effect of nalidixic acid on the incorporation of radioactive adenine and uracil into RNA and DNA. Incorporation into DNA was determined as follows: samples of cultures were removed to 0.1 volume of 5.5 N NaOH, and were held at 37 C for 15 hr; after acidification with 0.1 volume of 6 N HCl and 1.0 volume of 10% trichloroacetic acid in the cold, the precipitate was collected by membrane filtration for radioisotopic assay. Incorporation into RNA was determined as the difference between total incorporation (acid insolubles) and the incorporation into DNA.

RESULTS

Effect of nalidixic acid on the synthesis of protein, RNA, and DNA in E. coli 15TAU. Earlier, it was shown by direct chemical analysis of treated cultures (Goss et al., 1964) that nalidixic acid affects DNA synthesis in *E. coli*. This inhibition of DNA synthesis by nalidixic acid has been confirmed and studied in detail by radioisotopic tracer techniques.

Synthesis of DNA, protein, and RNA in *E. coli* 15TAU can be studied by following the incorporation of radioactive precursors (thymine, L-arginine, and uracil), without complications arising from endogenous precursor synthesis.

Radioactive thymine, L-arginine, or uracil (0.1 μc/ml, final concentration) was added to separate portions of an exponentially growing culture. Each portion was divided, and nalidixic acid (50 μg/ml, final concentration) was added to one part, the other serving as an untreated control. Samples were removed at intervals for determination of the radioactivity incorporated into the acid-insoluble fraction.

In the control cultures, the synthesis of DNA, protein, and RNA proceeded rapidly (Fig. 1). After 60 min of incubation, the amount of radioactivity incorporated into acid-insoluble material had increased 11- to 12-fold for each precursor.

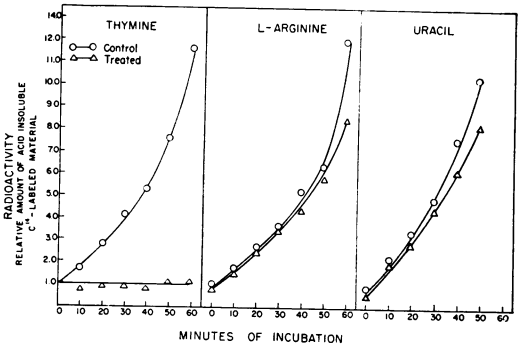

FIG. 1. *Effect of nalidixic acid (50 μg/ml) on the incorporation of C^{14}-labeled thymine, L-arginine, and uracil into the acid-insoluble fraction of Escherichia coli 15TAU.*

In nalidixic acid-treated cultures, any apparent effects on protein and RNA synthesis during the first 40 min of incubation were questionable. By 60 min, however, some inhibition was evident. Incorporation of L-arginine into acid-insolubles was inhibited 28%, and uracil incorporation was inhibited 21%.

In contrast, cultures exposed to nalidixic acid failed to incorporate C^{14}-labeled thymine into DNA; this inhibition was evident within 10 min (Fig. 1). At a level of 50 μg/ml, which is approximately 20 times the minimal growth inhibitory concentration, nalidixic acid blocked the incorporation of thymine rapidly and completely.

Inhibition of DNA synthesis in E. coli 15TAU by nalidixic acid. The inhibition of DNA synthesis in the presence of various levels of nalidixic acid was examined by use of C^{14}-labeled thymidine as the precursor. Cultures of *E. coli* were exposed to 1.0, 3.0, and 10.0 μg/ml of nalidixic acid in the presence of C^{14}-labeled thymidine (5×10^{-3} μc/ml, final concentration). It had been shown previously that 1.0 μg/ml inhibited growth, whereas 10.0 μg/ml were lethal to proliferating cultures of *E. coli* 15TAU (Goss et al., 1964).

There was rapid uptake and incorporation of thymidine into the acid-insoluble fraction in the control culture and in the culture exposed to 1.0 μg/ml of nalidixic acid (Fig. 2). However, the incorporation of thymidine was inhibited 30 to 40% by exposure to 3.0 μg/ml (slightly greater than the minimal growth inhibitory concentration). At 10.0 μg/ml, the incorporation was inhibited 72%.

Inhibition of DNA synthesis in E. coli 198 by nalidixic acid. It was conceivable, however, that the observed inhibitory effect on thymine (or

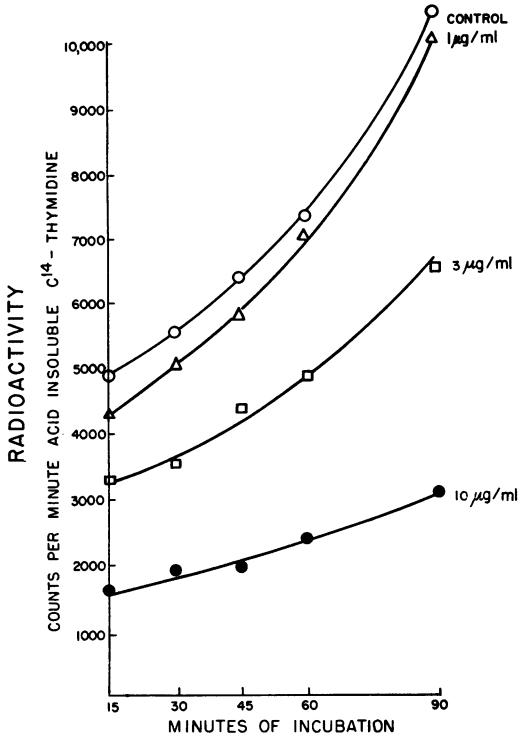

FIG. 2. *Effect of the concentration of nalidixic acid on the incorporation of C^{14}-labeled thymidine into the acid-insolubles of Escherichia coli 15TAU.*

thymidine) incorporation could be due to some peculiarity of the strain of *E. coli* used. For example, the uptake and intracellular transport of thymine might be abnormal in this thymineless polyauxotroph.

To distinguish between a block in the uptake of precursors and the actual synthesis of DNA, one can study the incorporation of a precursor common to both RNA and DNA. By comparing the incorporation of the purine, adenine, and the pyrimidine, uracil, one also can rule out a specific effect of the drug on pyrimidines.

We have examined the effect of nalidixic acid on the incorporation of both adenine and uracil into RNA and DNA of *E. coli* 198, a wild-type strain. The specific inhibition of DNA synthesis was observed with both precursors. This indicates that this inhibition is not specific for purines or pyrimidines, and that it does not involve a block in the uptake of precursors.

Separate portions of cultures of *E. coli* 198 were exposed to nalidixic acid (1.0, 3.0, or 10.0 μg/ml) in the presence of C^{14}-labeled adenine or C^{14}-labeled uracil. Unlabeled adenine or unlabeled uracil was added to the basal glucose-salts medium to a concentration of 10.0 μg/ml. The acid-insoluble materials were chemically separated into the RNA and DNA fractions for isotopic assay.

Although *E. coli* 198 can proliferate without exogenously supplied adenine or uracil, this organism incorporates these bases into RNA and DNA very rapidly. Approximately 7 to 10 times as much labeled material was found in the RNA fraction as in the DNA fraction of cultures exposed to either radioactive adenine or uracil (Fig. 3).

Nalidixic acid at low levels (1.0 μg/ml) had no apparent effect on the incorporation of C^{14}-labeled adenine into RNA. However, incorporation into DNA was inhibited approximately 66% after 60 min of exposure to the drug (Fig. 3). At higher levels, nalidixic acid inhibited the incorporation of adenine into both RNA and DNA. However, this inhibitory effect was more pronounced in the DNA fractions at all drug levels (Table 1).

The results obtained with radioactive uracil were quite similar; there was little or no effect of nalidixic acid at 1.0 μg/ml on the uptake and incorporation into RNA. The incorporation of uracil into DNA was inhibited 69% after 60 min of incubation. At higher drug levels, incorporation into RNA was inhibited also, but this effect

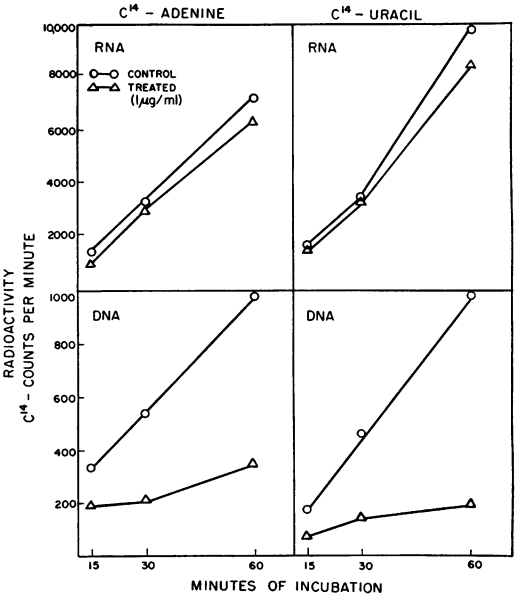

FIG. 3. *Effect of nalidixic acid (1.0 μg/ml) on the uptake and incorporation of C^{14}-labeled adenine and C^{14}-labeled uracil into RNA and DNA by Escherichia coli 198.*

TABLE 1. *Inhibition of RNA and DNA synthesis in Escherichia coli 198 by nalidixic acid**

C^{14}-labeled precursor	Concn of nalidixic acid (μg/ml)					
	1.0		3.0		10.0	
	RNA	DNA	RNA	DNA	RNA	DNA
Adenine.....	12.8	66.0	22.0	73.8	47.0	81.1
Uracil.....	13.6	69.0	21.0	65.0	40.0	74.0

* After 60 min of incubation, the radioactivity incorporated into the RNA and DNA fractions was determined by the modified Schmidt-Thannhauser technique of Roodyn and Mandel (1960). The results are expressed as per cent inhibition.

was less pronounced than that observed in the DNA fractions (Fig. 3, Table 1).

Effect of nalidixic acid on the initiation of DNA synthesis and on the DNA replication process. When *E. coli* 15TAU is transferred from a (+T, +AU) medium to a (+T, −AU) medium, DNA synthesis continues for approximately one generation time, then ceases. The total increase in the amount of DNA under these conditions is 40 to 60%. This has been attributed to a need for the synthesis of a specific protein referred to as "initiator." When a culture of *E. coli* 15TAU is transferred from a (+T, −AU) medium to a (−T, +AU) medium, the capacity to synthesize initiator is restored but DNA synthesis is prevented. Unrestricted DNA synthesis will proceed if the culture is subsequently transferred to the complete medium (+T, +AU). However, if protein synthesis is prevented by the addition of chloramphenicol during the incubation in the (−T, +AU) medium, then there will be a lag in DNA synthesis upon removal of the drug and subsequent transfer to the complete medium (+T, +AU). A similar lag is seen when cells are transferred directly to complete medium after incubation in (+T, −AU) medium (Nakada, 1960; Maaløe and Hanawalt, 1961). Thus, by appropriate manipulation of nutritional conditions, it is possible to separate the initiation and replication phases of DNA synthesis, and to study each individually.

Before examining the effect of nalidixic acid on the initiation of DNA synthesis, it was imperative to demonstrate that the capacity to synthesize DNA can be restored by removal of the drug.

DNA synthesis in *E. coli* 15TAU growing in a (+T, +AU) medium was blocked by the addition of 10 μg/ml of nalidixic acid. After 75 min, a portion of the treated culture was filtered and washed to remove the drug. These cells were then transferred to drug-free growth medium containing radioactive thymine. DNA synthesis was restored to approximately the same rate as the untreated control culture (Fig. 4).

Since it was then possible by removal of the drug to restore the capacity to synthesize DNA, we next examined the effect of nalidixic acid on "initiator." Under conditions in which chloramphenicol exhibited a distinct effect, no effect of nalidixic acid was demonstrated on the initiation of DNA synthesis.

A growing culture of *E. coli* 15TAU was allowed to complete the DNA replication cycle by incubation for 90 min in a (+T, −AU) medium. This culture was then transferred to a (−T, +AU) medium, and the optical density (OD) was adjusted to 0.100. Nalidixic acid (10 μg/ml) was added to one portion of the culture, chloramphenicol (10 μg/ml) to another, and a third was reserved as control. Incubation was continued for 30 min, at which time the OD was recorded. Each culture was filtered and washed with prewarmed basal medium. Next, each of these cultures was transferred to a (+T, +AU) medium, and the OD was readjusted to the value

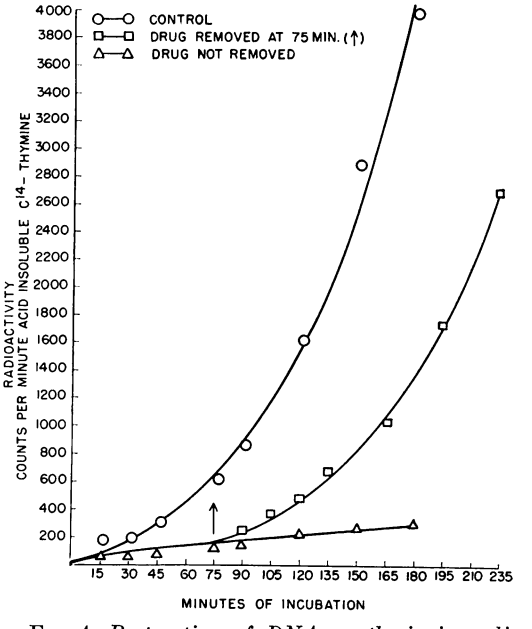

FIG. 4. *Restoration of DNA synthesis in nalidixic acid-treated cultures. An exponentially proliferating culture of Escherichia coli 15TAU was treated with nalidixic acid (10 μg/ml). After 75 min of incubation, nalidixic acid was removed from a portion of the treated culture by membrane filtration. These cells were washed and resuspended in drug-free growth medium containing radioactive thymine (5×10^{-3} μc/ml, final concentration). Acid-insolubles were collected at the times indicated.*

recorded prior to filtration. Finally, radioactive thymine (5×10^{-3} μc/ml, final concentration) was added to each culture. Acid-insolubles were collected, and OD values were determined at the times indicated.

During the 30 min of incubation in the (−T, +AU) medium, the turbidity (which one may equate with cell mass) of control and nalidixic acid-treated cultures increased about 45%. In contrast, the turbidity of the culture treated with chloramphenicol increased only 14% (Fig. 5).

On transfer to the (+T, +AU) medium, the increase in turbidity and incorporation of radioactive thymine into DNA proceeded without detectable lag in both the control culture and the culture previously treated with nalidixic acid. However, in the culture previously treated with chloramphenicol there was a definite lag in both turbidity increase and thymine incorporation (Fig. 5).

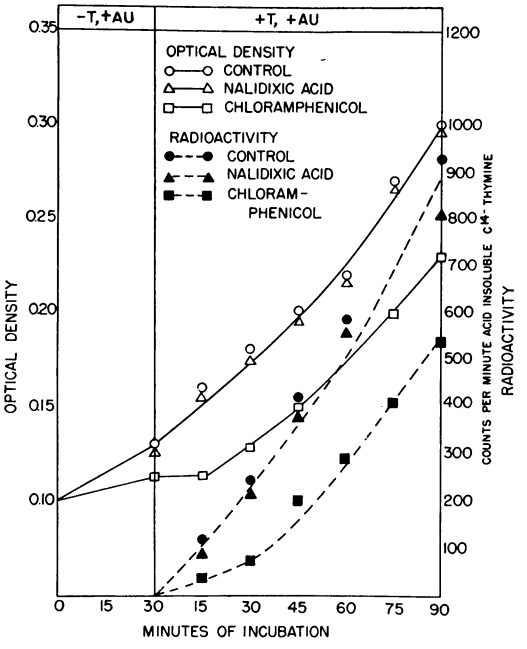

FIG. 5. *Effect of nalidixic acid on protein and RNA synthesis required for the initiation of DNA replication. Escherichia coli 15TAU was transferred from a (+T, +AU) medium to a (+T, −AU) medium. After 90 min of incubation, this culture was transferred to a (−T, +AU) medium. Nalidixic acid (10 μg/ml) and chloramphenicol (10 μg/ml) were added to separate portions of the culture; one portion was reserved as control. Incubation was continued for 30 min, at which time each culture was transferred to a (+T, +AU) medium containing radioactive thymine (5×10^{-3} μc/ml, final concentration). Acid-insolubles were collected, and the OD values determined at the times indicated.*

Effect of nalidixic acid on the DNA replication process in the absence of protein and RNA synthesis. Finally, we examined the effect of nalidixic acid on a single round of DNA replication in the absence of RNA and protein synthesis. Under these conditions, nalidixic acid blocked DNA replication, but did not result in loss of viability.

DNA synthesis was monitored by following the incorporation of C^{14}-labeled thymine into acid-insolubles. Turbidity was used to follow changes in cell mass (RNA and protein).

An exponentially proliferating culture of *E. coli* 15TAU was transferred by membrane filtration to a (+T, −AU) medium containing C^{14}-labeled thymine (5×10^{-2} μc/ml, final concentration). Nalidixic acid (20 μg/ml) was added 15 min after the addition of radioactive thymine. Samples were removed from cultures for radioactivity and viability determinations at the times indicated.

Over the course of the experiment, the OD of the control culture increased very slightly (0.100 to 0.112), indicating that increase in cell mass (RNA and protein synthesis) was prevented in

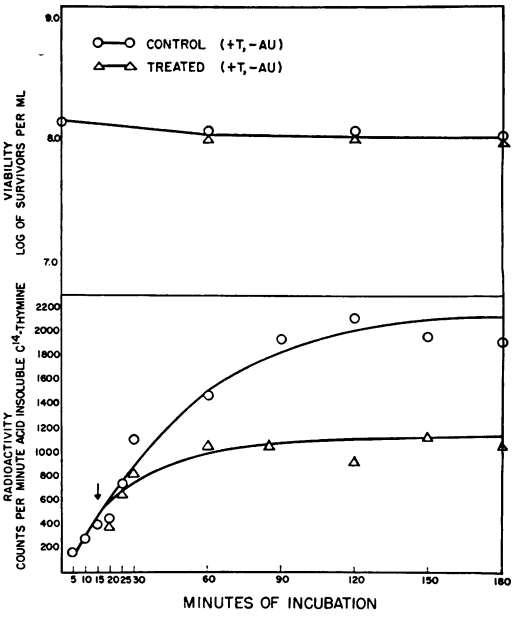

FIG. 6. *Inhibition of DNA synthesis in the absence of RNA and protein synthesis. A culture of Escherichia coli 15TAU was transferred from a (+T, +AU) medium to a (+T, −AU) medium containing C^{14}-labeled thymine. Nalidixic acid (20 μg/ml) was added (arrow) 15 min after addition of radioactive thymine.*

TABLE 2. *Relationship of thymineless death (TLD) and the action of nalidixic acid in the presence and absence of protein and RNA synthesis**

Medium	Per cent mortality at 3 hr†	Interpretation
Thymineless media		
(−T, +AU)	99.9	Loss of viability attributable to thymine deficiency (TLD) in the presence of protein and RNA synthesis
(−T, −AU)	67	Loss of viability attributable to thymine deficiency (TLD) in the absence of protein or RNA synthesis
(−T, +AU) + chloramphenicol	67	Loss of viability attributable to thymine deficiency (TLD) in the absence of protein or RNA synthesis
Thymine media		
(+T, +AU) + nalidixic acid	99.7	Loss of viability attributable to nalidixic acid in the presence of protein and RNA synthesis
(+T, −AU) + nalidixic acid	8	No loss of viability attributable to nalidixic acid in the absence of protein or RNA synthesis
(+T, −AU) − nalidixic acid	25	No loss of viability in the absence of RNA or protein synthesis.

* An exponentially proliferating culture of *E. coli* 15TAU was transferred from (+T, +AU) medium to the media described above. Nalidixic acid (20 μg/ml) and chloramphenicol (10 μg/ml) were added immediately after the transfer.

† Calculated from the initial number of viable bacteria at zero-time (1.4×10^8/ml).

this medium. Radioactive thymine was incorporated rapidly in the control culture during the first 40 to 50 min of incubation. Thereafter, the rate of incorporation decreased considerably (Fig. 6). There was no appreciable change in the viability of the control culture under these conditions.

The addition of nalidixic acid at 15 min inhibited the incorporation of radioactive thymine into acid-insolubles. This inhibitory effect was observed after 15 to 30 min of exposure to the drug. Although DNA synthesis was inhibited by nalidixic acid under these conditions (+T, −AU), there was no appreciable change in the bacterial viability (Fig. 6). Microscopic examination of treated cultures after 3 hr of incubation revealed morphologically normal cells.

Comparison of thymineless death and the action of nalidixic acid. We have shown that the action of nalidixic acid in a complete medium resembles thymineless death (Goss et al., 1964). However, the requirements for lethal action in the two processes are not identical.

Barner and Cohen (1957) reported that, in strains of *E. coli* requiring both thymine and an amino acid, thymineless death is markedly impeded when both requirements are omitted from the medium. With *E. coli* 15TAU, thymineless death occurs to a limited extent in a (−T, −AU) medium (Kanazir et al., 1959; Maaløe, 1961). Thymineless death is also restricted but not prevented in a thymineless strain of *E. coli* B when chloramphenicol is present in the medium (Gallant and Suskind, 1961).

Although the lethal action of nalidixic acid on *E. coli* 15TAU is similar in some respects to thymineless death, the following study points out a distinct difference between the two phenomena. In a medium permitting RNA and protein synthesis (−T, +AU), 99.9% of *E. coli* 15TAU cells lost viability during the 3-hr observation period (Table 2). It was confirmed that, by restricting protein and RNA synthesis in a (−T, −AU) medium or in a (−T, +AU) medium containing chloramphenicol, a smaller portion of the total cell population underwent thymineless death (67% loss of viability).

Nalidixic acid was lethal to *E. coli* 15TAU in a medium permitting protein and RNA synthesis (+T, +AU); approximately 99.7% of the cells lost viability during the 3 hr. In contrast, a lethal effect attributable to nalidixic acid was not observed when both protein and RNA synthesis were prevented (+T, −AU). These observations confirm that nalidixic acid does not have a lethal effect on nonproliferating cells of *E. coli*.

DISCUSSION

The results of these studies, as well as those reported previously (Goss et al., 1964), demonstrated an inhibition of DNA synthesis by nalidixic acid. To consider this inhibition of DNA synthesis as a primary action on *E. coli*, two criteria should be met: the biochemical effect

and the lethal effect must be correlative; and the concentration of nalidixic acid required to produce the biochemical effect must approximate the minimal bactericidal concentration.

These criteria have been met. The incorporation of precursors into DNA is inhibited by nalidixic acid prior to a loss in viability. The concentration required to cause an inhibition in DNA synthesis approximates the minimal bactericidal level. The inhibition in DNA synthesis is the first observed biochemical effect and by far the most pronounced in degree.

By examining the effect of nalidixic acid on the incorporation of adenine and uracil into RNA by a wild-type strain of *E. coli*, we excluded any strain-specific effect of the drug on the uptake of precursors. In the presence of nalidixic acid, both bases were incorporated into RNA but not into DNA. This also excludes any selective effect of the drug on purines and pyrimidines. Because RNA synthesis continued in the presence of nalidixic acid at levels which inhibited DNA synthesis, the formation of the purine and pyrimidine bases and their conversion to the corresponding ribonucleotides can be eliminated as primary sites of action.

Finally, we have demonstrated that nalidixic acid has little, if any, effect on the synthesis of the "initiator" of DNA replication (Maaløe and Hanawalt, 1961). Chloramphenicol, on the other hand, has a pronounced effect on the formation of initiator.

The specificity of action of nalidixic acid is well demonstrated by examining its effect on a single round of DNA replication. Under conditions of restricted RNA and protein synthesis, DNA synthesis is inhibited by nalidixic acid without any detectable loss of bacterial viability, at least during a 3-hr exposure period. The lack of lethality in this system strongly suggests that nalidixic acid does not alter the biological integrity of preformed DNA, but rather prevents only the synthesis of new DNA.

The fact that cells exposed to nalidixic acid for as long as 75 min will recover from a block, or at least continue to incorporate thymine after the drug is removed, indicates that it is not firmly bound.

We have demonstrated previously that thymineless death and the action of nalidixic acid on *E. coli* 15TAU under conditions permitting RNA and protein synthesis are very similar (Goss et al., 1964). One point clearly distinguishes the two phenomena. Thymineless death occurs even in the absence of RNA and protein synthesis. In contrast, nalidixic acid has no lethal effect when RNA and protein synthesis are restricted.

The data presented here leave little doubt that a primary action of nalidixic acid on *E. coli* is inhibition of the biosynthesis of DNA. Investigations are in progress to examine the effect of nalidixic acid on the enzymes involved in DNA synthesis.

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